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CELL BIOLOGY & MOLECULAR GENETICS

AFLP Analysis of DNA from Dried Hop Cones

M. Shaun Townsend, John A. Henning,* and Daniel L. Moore

ABSTRACT

Hop (*Humulus lupulus* L.) cones are used extensively in beer brewing to enhance flavor and impart bittering. Verifying hops cultivar identity has traditionally been accomplished by morphological characteristics or a chemical analysis of lupulin glands but these traits may vary according to environmental influences. The objective of this research was to develop an AFLP (amplified fragment length polymorphism) protocol for analyzing DNA extracted from dried hop cones. The DNA was extracted from dried cones of six hop genotypes by a technique published for grapes (*Vitis* spp). The reagents, *Mse*I primers, and protocol were part of a commercially available kit, while the 6-carboxyfluorescein-labeled *Eco*RI primers were purchased separately. Eleven primer combinations amplified an average of 546.5 scorable fragments with an average of 49.7 fragments per primer combination. All genotypes were differentiated with the primer combinations studied. Average genetic similarity estimates ranged between 0.956 and 0.995 among the six hop genotypes studied. This research provides the hops industry with a powerful technique to verify accurately hops cultivar identity and purity through an analysis of dried cone DNA.

HOPS ARE USED in beer brewing to impart bittering, enhance flavor, and as a preservative. The plant organs of interest to brewers are the female flowers, or cones, which contain lupulin glands with a chemical profile desirable for brewing. The wide variation in chemical profiles found among hop cultivars gives the brewer flexibility to impart specific flavor and aroma characteristics to beer (Neve, 1991).

One of the challenges faced by both the brewing industry and hop breeders has been obtaining a reliable

technique to identify accurately hop cultivars. Traditionally, hops cultivar identification has been verified by either a chemical analysis of lupulin glands, or by morphological characteristics. Both traits are influenced by environmental conditions during cone development which, in turn, can lead to difficulty in cultivar identification (Hartl and Seefelder, 1998). Since hop cultivars are clonally propagated and all individuals within a cultivar are genetically identical, DNA fingerprinting using a molecular marker approach would be an ideal way to distinguish among hop cultivars.

The AFLP (amplified fragment length polymorphism) technique (Vos et al., 1995) is a recent innovation that has proven both powerful and reliable. This technique has several advantages over earlier marker technologies: very little genomic DNA is required, many polymorphisms can be generated per reaction, prior knowledge of specific DNA sequences is not needed, and the technique has high reproducibility (Vos et al., 1995). In a comparison among AFLP, RFLP (restriction fragment length polymorphism), and RAPD (random amplified polymorphic DNA) analysis of 14 soybean [*Glycine max* (L.) Merr.] cultivars, Lin et al. (1996) reported AFLP to average six polymorphic bands in 60 of the 64 primer pairs tested, RAPD produced a polymorphic band with only 35% of the primers tested, and more than 50% of the RFLP probes failed to distinguish a polymorphic band. In other work, Lu et al. (1996) found that AFLP generated more polymorphic bands per primer or probe than other molecular marker technologies.

The AFLP technique has been successfully used to differentiate genetically populations or individuals within numerous species including tea (*Camellia sinensis* L.) (Paul et al., 1997), cotton (*Gossypium barbadense* L.) (Feng et al., 1997), tef (*Eragrostis tef* (Zucc) Trotter)

M.S. Townsend, Dep. of Crop and Soil Sci. Oregon State Uni., Corvallis, OR 97331-3002; J.A. Henning and D.L. Moore, USDA-ARS, Corvallis, OR 97331-3002. Contribution of the USDA-ARS. Received 15 Nov. 1999. *Corresponding author (John.Henning@orst.edu).

Table 1. Primer sequences used for AFLP analysis of DNA extracted from dried hop cones.

<i>Eco</i> RI		<i>Mse</i> I
	<u>Adaptors</u>	
5-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5		5-GACGATGAGTCCTGAG TACTCAGGACTCAT-5
	<u>Core Primers</u>	
5-GACTGCGTACCAATTC		5-GATGAGTCCTGAGTAA
	<u>Preselective Amplification Primers</u>	
Core Primer + A		Core Primer + C
	<u>Selective Amplification Primers</u>	
Core Primer + AAC + FAM label		Core Primer + CAA
Core Primer + ACC + FAM label		Core Primer + CAC
Core Primer + AGC + FAM label		Core Primer + CAG
		Core Primer + CAT
		Core Primer + CTA
		Core Primer + CTC
		Core Primer + CTG
		Core Primer + CTT

(Bai et al., 1999), soybean (Lin et al., 1996), and wheat (*Triticum aestivum* L.) (Barrett and Kidwell, 1998). Hartl and Seefeldt (1998) used AFLP to evaluate DNA from leaf tissue of eight hop cultivars for genetic diversity. They obtained 523 AFLP fragments from eight primer combinations, and the average polymorphism count per primer combination was 18. They were unable to distinguish between three of the hop cultivars, even when an additional 50 primer pairs were used. Conversely, Schut et al. (1997) were able to identify 31 barley lines with only eight AFLP primer combinations. They obtained 681 markers with over 43% showing polymorphism, and each primer combination was able to discriminate all of the 31 barley lines studied.

Hop cones are dried and processed on farm before delivery to marketing agents and/or a brewer. We are not aware of any research reports describing the use of AFLP analysis of DNA from dried hop cones. A technique for analyzing hop cone DNA would allow a brewer to verify accurately a hop sample's purity and identity prior to the brewing process. The objective of this research was to develop an AFLP protocol for analyzing DNA from dried hop cones.

MATERIALS AND METHODS

Plant Material and DNA Extraction

Hop genotypes analyzed were the cultivars Sterling (USDA-21689), Nugget (USDA-21193), Wye Target (USDA-21112), Hallertauer Gold (USDA-21671), and the genotypes USDA-21688 and USDA-21737. The genotype USDA-21688 is a half-sib sister to Sterling, and USDA-21737 is a virus-free Sterling hops that was propagated from meristem culture. Hop cones were dried in a forced-air drier at 65°C prior to analysis. The experiment was repeated twice beginning with DNA extraction.

The DNA extraction protocol used was a slight modification of a protocol reported for grapes (Lodhi et al., 1994). A 0.5-g sample of dried hops cone tissue was ground in liquid nitrogen with a mortar and pestle. After grinding, the sample was transferred to a sterile 20-mL polypropylene tube and 6 mL of pre-heated (65°C) CTAB extraction buffer [100 mM Tris-HCL, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB (w/v)], and 50 mg polyvinylpyrrolidone was added and the capped tube was gently inverted several times to mix the contents.

Samples were incubated at 65°C for 30 min and allowed to cool at room temperature for 10 min. During incubation, the tubes were gently inverted several times every 10 min. After incubation, 7 mL of chloroform:isoamyl alcohol (24:1) was added, samples were inverted approximately 20 times, and then centrifuged at $2600 \times g$ for 15 minutes. The upper aqueous phase was transferred to a new sterile 20-mL polypropylene tube by means of a wide-bore pipette tip. An equal volume of 5 M NaCl and 2 volumes of cold (−20°C) 95% ethanol (v/v) were added. The tubes were inverted several times and refrigerated (4°C) for 60 min. Nucleic acids were pelleted at $3600 \times g$ (4°C) for 15 min. The supernatant was removed and the pellet was washed with cold (−20°C) 70% ethanol (v/v). The ethanol was evaporated by heating the samples to 37°C, the pellet dissolved in 600 µL of sterile TE buffer (pH 8.0), and the samples were treated with 10 units of RNase and stored in a refrigerator (4°C). The DNA was quantified with a Dynaquant 200 fluorometer (Hoefer Pharmacia Biotech, Inc., San Francisco, CA).

AFLP Analysis

The protocol, enzymes, most reagents, and the *Mse*I primers used were supplied in a kit (AFLP Analysis System I) manufactured by Life Technologies, Inc. (Rockville, MD). The 6-carboxyfluorescein-labeled (FAM) *Eco*RI primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Primers and DNA sequences used in this study are listed in Table 1. Approximately 250 ng of DNA was digested with 2.5 units each of *Eco*RI and *Mse*I endonuclease for 2 h at 37°C. *Eco*RI and *Mse*I adaptors were ligated to the DNA fragments using 1 unit of T4 DNA ligase for 2 h at 20°C. The ligated DNA fragments were diluted 1:10 with TE buffer. Preselective and selective PCR amplification reactions were performed in a Genius thermocycler (Techne, Inc., Princeton, NJ).

For preselective amplification, a 51-µL reaction mixture was used containing 5 µL of the diluted DNA with ligated adaptors, 40 µL pre-amp primer mix, 5 µL 10× PCR buffer (100 mM Tris-HCl at pH 8.3, 15 mM MgCl₂, 500 mM KCl), and 5 units of *Ampli*Taq DNA polymerase (PE Applied Biosystems, Foster City, CA). The preselective amplification PCR program was 20 cycles with the following program: 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. Following preselective amplification, samples were diluted 1:10 with TE buffer.

The total volume for the selective amplification reaction was 20.5 µL and contained 13.9 ng of FAM-labeled *Eco*RI primer, 30.15 ng of *Mse*I primer and dNTPs, 10 µL of enzyme

Table 2. Average number of AFLP fragments scored and polymorphic fragments detected for eleven primer combinations in hop cone DNA.

Primer combination	Fragments scored	Polymorphic fragments	Percent polymorphic
<i>eAAC-mCAC</i> †	50.5 ± 8.5‡	4.0 ± 0.0	18.7 ± 11.7
<i>eAAC-mCAG</i>	76.5 ± 6.5	10.0 ± 2.0	17.0 ± 6.2
<i>eAAC-mCTA</i>	49.0 ± 0.0	3.0 ± 0.0	7.1 ± 1.0
<i>eAAC-mCTC</i>	64.0 ± 3.0	8.5 ± 2.5	16.6 ± 7.6
<i>eACC-mCAC</i>	37.0 ± 7.0	6.5 ± 0.5	23.5 ± 7.6
<i>eACC-mCAG</i>	40.5 ± 1.5	4.0 ± 0.0	9.9 ± 0.4
<i>eACC-mCTA</i>	52.5 ± 0.5	4.0 ± 1.0	8.5 ± 2.8
<i>eAGC-mCAG</i>	49.5 ± 5.5	13.5 ± 0.5	31.8 ± 8.2
<i>eAGC-mCAT</i>	41.5 ± 0.5	3.5 ± 1.5	7.4 ± 2.6
<i>eAGC-mCTC</i>	43.0 ± 3.0	4.0 ± 1.0	10.3 ± 2.8
<i>eAGC-mCTG</i>	42.5 ± 3.5	1.5 ± 1.5	6.3 ± 6.3
Total	546.5 ± 13.5	62.5 ± 9.5	
Average	49.7 ± 1.2	5.7 ± 0.9	11.4 ± 2.2

† “e” denotes the *EcoRI* primer while “m” denotes the *MseI* primer.

‡ $N = 2$.

solution (79 μ L H₂O, 20 μ L 10 \times PCR buffer, 5 units *Ampli-Taq*), and 5 μ L of diluted DNA template from the preselective amplification reaction. The first 10 PCR cycles used a step-down of the annealing temperature with each cycle: 94°C for 60 s, 65°C (initial) for 60 s, and 72°C for 90 s. The annealing temperature was decreased by 1°C with each cycle. The final 23 cycles were run at a constant annealing temperature: 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s.

Gel bands were detected on an ABI 377 DNA sequencer (PE Applied Biosystems) and scored by Genographer software (Benham et al., 1999). Within each DNA extraction, two AFLP runs were performed and only those fragments present in both runs were scored. A binary data matrix was created from the scored images and genetic similarities computed according to Dice (1945) as $GS_{ab} = 2M_{ab} / (P_a + P_b)$, where GS_{ab} is the genetic similarity between Genotypes *a* and *b*, M_{ab} is the number of common bands between Genotypes *a* and *b*, P_a is the number of bands in Genotype *a*, and P_b is the number of bands in Genotype *b*. Means and standard deviations were computed in the usual fashion.

RESULTS AND DISCUSSION

Initial results from replicated runs showed substantial variation in fragment patterns in certain genotypes with a given primer combination. Upon checking the digested DNA on an agarose gel, we discovered that the DNA extracted from hop cones was not being completely digested by the restriction endonucleases. Apparently, contaminants in the DNA extracted with the original procedure (Doyle and Doyle, 1990) we used were inhibiting restriction digestion. A DNA extraction procedure that reportedly works well with *Vitis* spp. (Lodhi et al., 1994) was tried and the genomic DNA was thoroughly digested. The resulting AFLP fragment patterns were much less variable among runs.

One of the primer combinations (*eACC-mCTT*) failed to amplify fragments in three of the genotypes and was omitted from the study. The 11 remaining primer combinations amplified 546.5 scorable DNA fragments with an average of 49.7 fragments per primer combination (Table 2). The primer combination *eACC-mCAC* produced the fewest number of scorable fragments while *eAAC-mCAG* produced the most. Overall, an average of 62.5 total polymorphisms were detected with an average of 5.7 polymorphisms per primer combination. Using AFLP analysis in soybean, Lin et al. (1996) detected 50 to 100 bands and an average of 5.6 polymorphism per primer pair. Hartl and Seefelder (1998) reported that eight primer pairs amplified 523 DNA fragments and averaged 18 polymorphisms in hop leaf tissue. The difference in total fragments amplified and polymorphisms detected between our work and that of Hartl and Seefelder (1998) could be due to the different primer combinations used in each experiment, or due to the different plant tissues sampled (Donini et al., 1997). We only analyzed DNA extracted from dried cone tissue while Hartl and Seefelder (1998) only analyzed DNA extracted from hop leaf tissue.

Primer combinations *eAAC-mCAG* and *eAGC-mCAG* generated the most polymorphisms while the fewest polymorphic fragments were detected with *eAAC-mCTA*, and *eAGC-mCTG* (Table 2). Across the *MseI* primers evaluated, the primer combinations containing the *EcoRI* primer AAC averaged 60 scorable fragments and 6.4 polymorphic fragments while the combinations containing the *EcoRI* primer ACC averaged 43.3 scorable fragments and 4.8 polymorphic fragments. Although primer combinations based upon the *EcoRI* primer AAC averaged the highest number of scorable and polymorphic fragments, this may not have been the case if the same group of *MseI* primers had been used for each of the *EcoRI* primers studied. Primer combinations *eAAC-mCTA*, *eACC-mCTA*, and *eAGC-mCAT* were the least variable between runs for total fragments scored. No variation for polymorphic fragments was detected between runs for the primer combinations *eAAC-mCAC*, *eAAC-mCTA*, and *eACC-mCAG* (Table 2). Some variation among replicated AFLP runs for scorable fragments has been reported in other work (Hartl and Seefelder, 1998; Zhang et al., 1999).

Average genetic similarity estimates ranged between 0.956 and 0.995 among the six hop genotypes studied, and there was some variation between runs for all of the estimates (Table 3). Sterling and USDA-21737 were the most closely related with an average of 4.5 polymor-

Table 3. Average genetic similarity estimates and standard deviations across 11 primer combinations based upon AFLP analysis of hop cone DNA.†

	Sterling	USDA 21688	Nugget	Hallertauer Gold	Wye Target
USDA-21688	0.991 ± 0.008				
Nugget	0.976 ± 0.024	0.972 ± 0.023			
Hallertauer Gold	0.986 ± 0.012	0.981 ± 0.012	0.973 ± 0.021		
Wye Target	0.963 ± 0.020	0.956 ± 0.022	0.963 ± 0.031	0.958 ± 0.027	
USDA-21737	0.995 ± 0.007	0.986 ± 0.012	0.972 ± 0.028	0.987 ± 0.011	0.960 ± 0.024

† Computed according to Dice (1945).

‡ $N = 11$ for each estimate.

phic fragments between them. Since USDA-21737 is Sterling hops that has undergone meristem culture, the genetic difference between the two genotypes may have arisen during the meristem culture process, or from the presence of viral DNA in Sterling. Most of the primer combinations studied were able to differentiate between the half-sib sisters Sterling and USDA-21688 with an average of 8.5 polymorphic fragments detected. These data show that closely related individuals in hops can be readily differentiated with an AFLP analysis of dried cone DNA as long as suitable primer combinations are employed.

The lowest genetic similarity estimates observed were between Wye Target and the other genotypes studied (Table 3), which corroborates the work of Hartl and Seefelder (1998). Although Hartl and Seefelder (1998) studied different hop cultivars and used different primer pairs, some data similarities were evident between our work and theirs. Average genetic similarities in our work for comparisons between Sterling vs. Nugget (0.976), Sterling vs. Hallertauer Gold (0.986), Nugget vs. Hallertauer Gold (0.973), and Nugget vs. Wye Target (0.963) were comparable to Saazer vs. Northern Brewer (0.95), Saazer vs. Hallertauer Mittelfruher (0.98), Northern Brewer vs. Hallertauer Mittelfruher (0.95), and Northern Brewer vs. Wye Target (0.93) in Hartl and Seefelder's (1998) work. Although the genotypes used in the two studies are not identical, some are closely related. Sterling was derived from Saazer 38, both Nugget and Northern Brewer are predominantly Brewer's Gold, and Hallertauer Gold was derived from Hallertauer Mittelfruher (Al Haunold, 1999, personal communication; Neve, 1991). Our data suggest that AFLP will provide consistent results in hop cone DNA fingerprinting and genetic relationship research.

In conclusion, we report a method for employing AFLP analysis of DNA from dried hop cones. The AFLP technique generates many scorable DNA fragments which increases the chances of detecting genetic differences between closely related hop genotypes. All of the primer combinations used in this research were informative, and the combinations involving *eAAC* provided a greater number of scorable and polymorphic fragments across the *MseI* primers used here. Some variation between runs was observed for number of fragments scored and polymorphic fragments. This technique should prove useful for those needing to verify

hops identity and purity by providing a method of analyzing DNA extracted from dried hop cones.

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